

*Felix Mitelman*

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THE CHROMOSOMES  
OF  
ROUS RAT SARCOMAS

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LUND 1972





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This thesis is based on the following papers:

- I. Mitelman, F. and Mark, J. 1970. Chromosomal analysis of primary and metastatic Rous sarcomas in the rat. - *Hereditas* 65: 227-235.
- II. Mitelman, F. 1971. The chromosomes of fifty primary Rous rat sarcomas. - *Hereditas* 69: 155-186.
- III. Levan, G., Mitelman, F., Nichols, W.W., Beckman, G. and Beckman, L. 1971. Isozyme patterns in Rous sarcoma virus-induced tumors in the rat. - *Hereditas* 68: 143-150.
- IV. Mitelman, F. 1972. Comparative chromosome analysis of primary and metastatic Rous sarcomas in rats. - *Hereditas* 70: 1-14.
- V. Mitelman, F. 1972. Predetermined sequential chromosome changes in serial transplantation of Rous rat sarcomas. - *Acta Pathol. Microbiol. Scand. Section A*. In press.

Reference to these papers will be made by using the Roman numerals listed above. Some additional data from recent experiments described in this thesis are presented on pages 30-31.





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## INTRODUCTION

The role of chromosome changes in the aetiology of neoplasia has been a matter of debate since VON HANSEMANN (1890) and BOVERI (1914) suggested that abnormalities of the mitotic process and of the chromosome constitution were essential characteristics of the neoplastic cell. The chromosome findings, reviewed several times (LEVAN and BIESELE 1956; LEVAN 1959, 1967, 1969; KOLLER 1960, 1964, 1966; HAUSCHKA 1961, 1963; HSU 1961, 1965; STICH 1963; GUNZ and FITZGERALD 1964; HUGHES 1965; LEJEUNE 1965, 1967; NOWELL 1965; BAIKIE 1966; DE GROUCHY 1966; MILES 1966; SANDBERG 1966; CONEN 1967; JEAN and BOIS 1967; CONEN and ERKMAN 1968; DE GROUCHY and DE NAVA 1968; BERGER 1969; GOTTLIEB 1969; PORTER et al. 1969; SPRIGGS 1969; ATKIN 1970; SANDBERG and HOSSFELD 1970; STICH and YOHAN 1970), have by some of the authors been interpreted as causative factors essential for the initiation of the malignant state, whereas others have dismissed them as epiphenomena in tumour progression. Thus far, the only unequivocal correlation between a specific type of malignancy and a specific chromosome aberration is that between the Philadelphia chromosome and chronic myelogenous leukaemia (CML). Accumulating data, however, suggest that the chromosome variation, although speciously haphazard, in most carefully analyzed tumours seems to be governed by strict rules; the distinction between CML and other malignancies thus being more in degree than in kind. Moreover, chromosome patterns actually existing in specific tumour types may elude detection for various reasons:

1. The chromosomal state in neoplasia is dynamic. An early chromosome aberration may therefore be obscured by superimposed changes with more indistinct patterns common to progressed tumours of many different types.

2. The patterns are vague. These may become discernible only after many tumours of the same kind have been analyzed.

3. The histologic classification of neoplasia is crude. Tumours with the same pattern may be produced by different oncogenic mechanisms, each with its specific karyotypic changes.

4. No simple standard technique is available for the study on chromosomes of tumour cells.

In line with the above considerations, our group has concentrated for several years on chromosome studies in direct fixations of primary tumours with uniform aetiology - the Schmidt-Ruppin strain of Rous chicken sarcoma virus (RSV-SR) - in several different rodent species. Two comprehensive investigations within this project have been concluded: the studies by MARK (1967 b) of 91 tumours in the mouse and by KATO (1968) of 42 tumours in the Chinese hamster. The present investigation on 63 primary RSV-SR-induced rat sarcomas is another contribution to this field.

#### AIM OF THE INVESTIGATION

The aims of the investigation were to determine -

1. the chromosomal pattern of primary Rous rat sarcomas;
2. the chromosomal pattern in different regions of the same primary sarcoma;
3. the isozyme patterns in relation to the chromosomes in primary sarcomas;
4. the relation between latency period, tumour age and chromosomes in primary sarcomas;



5. the chromosomal relationship between primary sarcomas and their metastases;
6. the chromosomal progression during in vivo transplantation of several series of the same primary sarcoma;
7. the relationship between the chromosomes and the histopathologic picture in primary, metastatic and transplanted sarcomas.

#### MATERIAL AND METHODS

The rats used belonged to the same inbred Wistar/Furth (W/Fu) albino strain propagated at our institute by strict brother-sister mating in a continuous single line.

Sarcomas were induced by Rous chicken sarcoma virus, strain Schmidt-Ruppin (RSV-SR). A detailed presentation of the material is given in papers I and II; for other details concerning induction and spread of Rous rat sarcomas, see AHLSTRÖM and JONSSON (1962) and SALDEEN (1963), respectively. The titre of the virus inoculation was determined according to TEMIN and RUBIN (1958).

The chromosomes were studied in direct fixations from the tumour according to the procedure described in paper I. Whenever possible, the chromosomes of 50 or more metaphases were counted, and in none of the tumours less than 20 metaphases. Ten cells or more were karyotyped from each stemline and 5 cells or more from each sideline. Karyotype analyses were made by photography, or in many cases by drawing with the aid of camera lucida. The normal karyotype of W/Fu rats was studied in direct fixations from femur marrow of adult rats and also in fixations from tissue cultures of embryonic and adult tissues by slight modifications of the squash techniques of TJIO and WHANG (1962) and HSU and KELLOGG (1960).

The samples used for electrophoresis were prepared and examined by a method described in paper III. The following enzyme systems were studied: acid phosphatase, alkaline phosphatase, amino acid naphthyl amidase, peptidases, esterases, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and lactate dehydrogenase (LDH).

Tumour samples for histopathologic examination, as well as pieces of the lungs, liver, spleen and regional and para-aortic lymph nodes were stained routinely with haematoxylin-eosin and van Gieson.

#### TERMINOLOGY

The nomenclature and system of classification of the rat chromosomes follow LEVAN et al. (1964). Thus on the basis of centromeric location, the complement can be divided into 3 characteristic groups: the m group comprising 7 pairs with a median centromere, the st group consisting of 5 identifiable pairs with a submedian-subterminal centromere, and the t group comprising 9 pairs with a terminal or near-terminal centromere, the longest pair ( $t_1$ ) and the smallest chromosome (Y) being identifiable.

As in MARK (1967 a, b), S symbolizes the stemline, i.e. the most frequent karyotype of the tumour cell population, and s ( $s_1$ ,  $s_2$ , etc.) the sideline(s), i.e. other karyotypes of the population present in a frequency of 10% or more.

The term heteroploidy is used in a broader sense than the original one of WINKLER (see LEVAN and MÜNTZING 1963). In the

usual sense, heteroploidy signifies all chromosome numbers except those normally present in the regular alternation between the gametic and zygotic phases of typical representatives of a given species. In the present paper, this definition has been extended to cover structural deviations as well, and all karyotypes except the normal one of the species are referred to as heteroploid. Pseudodiploidy includes both diploidy with abnormal representation of morphologically normal chromosomes ("quasidiploidy" according to YERGANIAN et al. 1960) and diploidy with structurally changed chromosomes, both being included in the concept of heteroploidy.

## RESULTS

### The Normal Karyotype of W/Fu Rats (II)

The normal diploid chromosome number of Rattus norvegicus is 42. The karyotype was described in great detail by TJIO and LEVAN (1956), and FITZGERALD (1961) using newer techniques confirmed their findings. In 1963, HUNGERFORD and NOWELL showed that differences could occur in the morphology of the sex chromosomes among inbred strains and among population rats. Subsequently, autosomal polymorphism has been described among and within inbred strains of laboratory rats (YOSIDA and AMANO 1965; BIANCHI and MOLINA 1966; NOWELL et al. 1967; UDALOVA 1968; REES et al. 1968). Accordingly, it was necessary to study in detail the normal complement of the inbred W/Fu strain that was used in the present investigation.

In addition to numerous karyotype analyses of primary cultures from normal tissues of embryonic and adult animals, 600 me-

taphases from direct, bone-marrow preparations of 2 female and 3 male animals were analyzed. Except for mere products of doubling, all cells had the diploid number  $2n=42$ , and all karyotypes of both sexes agreed completely with that reported by TJIO and LEVAN (1956). No polymorphism, neither sex chromosomal nor autosomal, was observed in the W/Fu strain. As to diverging points in the published karyotypes, the following were observed: Both X's are t chromosomes that cannot be distinguished from the longer t autosomes, i.e. those in size following the longest t pair, which is an easily identifiable autosomal pair. The Y chromosome is the smallest t chromosome, often clearly heteropycnotic and always identifiable. Satellites are present on the  $st_2$  and  $st_5$  pairs.

#### The Chromosomal Pattern of Primary Rous Rat Sarcomas (I, II)

Sixty-three primary RSV-SR-induced rat sarcomas were analyzed in direct fixations. The results were in agreement with the only other such materials existing: 8 tumours studied by LEVAN (1961) and NICHOLS (1963) (6 in primary culture or first passage in vitro) and 9 tumours investigated cursorily in connection with other experiments by AHLSTRÖM and LEVAN (unpublished). Below, the pooled data of these 80 tumours will be used to characterize the chromosomal pattern of the Rous rat sarcomas.

#### Chromosome Numbers

About 80 per cent of the Rous rat tumours had diploid stemlines with a normal karyotype, half of them lacking sidelines and half possessing one or more sidelines. Hyperdiploidy was the most important heteroploid pathway: about 80 per cent of



the heteroploid stem- and sidelines were in the hyperdiploid region. Within this group, the trisomic number predominated with about 60 per cent of these stem- and sidelines. The trisomic number was, in fact, next in frequency after the diploid number. The second most common progressional pathway was pseudodiploidy, constituting some 12 per cent of the deviating stem- and sidelines, whereas hypodiploidy and polyploidy were of less importance, with 7 and 2 per cent of the deviating stem- and sidelines.

The general numeric pattern exhibited striking similarities with RSV-induced sarcomas of the mouse (MARK 1967 b) and the Chinese hamster (KATO 1968) - the only comprehensive studies existing on the chromosomes of Rous tumours in other animals. The much higher frequency of sarcomas with a normal diploid stemline in the rat as compared with the mouse and the Chinese hamster is probably due to differences in reaction inherent in the three species, as the optimal chromosome number region (ISING and LEVAN 1957). It may also be associated with the difference in karyotypic stability in culture between rat and mouse cells, the latter undergoing chromosomal alterations much earlier (JACKSON et al. 1970).

### Karyotypic Evolution

The chromosome variation in the development of the heteroploid stem- and sidelines was non-random. Thus, in 58 deviating stem- and sidelines, 43 displayed the same karyotypic feature, viz. 1 or more extra t chromosomes. This was seen most clearly among the trisomic stem- and sidelines, in which 1 extra t was the only change from normality in 24 out of 28 karyotypes. In the remaining 19 heteroploid stem- and sidelines with extra t chromosomes, additional changes had occurred. The most

frequent change, a gain of 1  $st_3$  chromosome, was observed in 13 of these karyotypes. Altogether, at least 1 extra  $t$  or  $st_3$  or both were found in 76 per cent of all heteroploid stem- and sidelines; but also in about 70 per cent of all variant cells. With the exception of three hypodiploid karyotypes, all stem- and sidelines without extra  $t$  or  $st_3$  chromosomes had undergone structural changes and possessed 1 or more markers. It should be noted, however, that at least some of the markers showed indications of having, in fact, originated from a medium-sized  $t$  chromosome.

The significance of the chromosomal deviations on the diploid level was tested statistically according to LEVAN (1966). The number expected for the 10 identifiable chromosome types was calculated for each stem- and sideline number, and the differences between found and expected were determined for each class. The material was divided into two groups, with and without markers. Among karyotypes without markers positive and significant differences were obtained for the  $t$  and  $st_3$  chromosomes. A corresponding significant but negative difference for chromosome type  $t$  was found among the stem- and sidelines with markers, supporting the hypothesis advanced above that most markers had originated from one of these chromosomes.

There were many indications that the karyotypic changes were stepwise and that the gain of  $t$  chromosomes preceded that of  $st_3$  chromosomes. Thus, the dominating variant cell in the diploid sarcomas without sidelines had 43 chromosomes, and three-fourth of these cells differed from the normal karyotype only by 1 extra  $t$  chromosome. This particular karyotype dominated all sidelines of the diploid sarcomas; and among the 10 sidelines that were earliest to appear, 8 had this karyotype. Among the diploid sarcomas with sidelines, the major part of the va-

riant cells manifested a development of the trisomic karyotype by a gain of 1  $st_3$  chromosome. With increasing age of the tumours, the latter karyotypes outgrew the trisomics. Thus, among the heteroploid sarcomas, karyotypes with 1 extra  $st_3$  were twice as frequent as the trisomic karyotypes with only one extra t. Also a third predetermined step was discernible: two tumour stemlines, and conspicuously many variant cells, in addition to the extra t and  $st_3$ , also showed one extra  $st_5$  chromosome (see p. 24).

In the Rous tumours of the mouse (MARK 1967 b), it was impossible because of the uniform chromosome morphology to determine whether any specific chromosome or chromosomes were preferentially involved in the heteroploid evolution. In the Chinese hamster (KATO 1968), on the other hand, it was demonstrated that the chromosome variation was non-random. As in the rat, this was most clearly seen among the trisomic karyotypes, but the variation was less restricted. Thus, in the Chinese hamster 3 chromosome types, i.e. chromosomes Nos. 5, 6 and 10, were represented as trisomics with almost equal frequency. It may be that in the rat different homologues of t chromosomes were involved in different cases. By careful aligning of the homologues, the extra t has always been classified as one of the medium-sized t chromosomes. Also, one or both arms of most markers corresponded to the length of a t chromosome of medium size, but unfortunately these cannot be individually identified so far, not even with autoradiographic methods (CHANG et al. 1965; BIANCHI 1966; BIANCHI and DE BIANCHI 1966; TAKAGI and MAKINO 1966; SASAKI 1971). The idea that the same t chromosome is involved in all cases therefore remains an assumption at present. Analysis by fluorescence staining is urgently needed for elucidating these questions.

The Chromosomal Pattern in Different Regions of the  
Same Primary Sarcoma (II)

The important concept of clonal evolution (FORD and CLARKE 1963; LEJEUNE et al. 1963; BERGER 1965; DE GROUCHY et al. 1966) has led to the generalization that malignant cells in any given sample of a tumour belong to the same clone. With this in mind, it was of interest to expand the present investigation to include a comparison of different tumour regions.

Eleven primary sarcomas were successfully analyzed in samples from 2 or 3 different regions. Nine sarcomas had identical stemlines in the different parts, in one case including a similar marker. Two sarcomas had different, but karyologically closely related stemlines in the different regions. With the dynamic equilibria among different elements of tumour cell populations, such differences are expected during the normal course of progression. None of the differences among samples from the same tumour indicated necessarily a polyclonal origin. Furthermore, the presence of a characteristic marker in two samples from the same tumour is a good indication of a common clonal origin of these parts of the tumour.

By means of biochemical techniques, strong evidence in favour of a single cell origin of tumours has been provided for uterine leiomyomas, CML and multiple myeloma, whereas the findings in a variety of other human neoplasms were suggestive of a polyclonal origin (for recent reviews, see FIALKOW 1970; OHNO 1971). The results of the present investigation offer neither evidence for nor against a single cell origin. The similar findings in different regions of the same tumour do, however, support the concept that progression is clonal, i.e. originating from a single mutant cell.

The study of different tumour regions led to another interesting observation. The necrotic centre of two sarcomas were successfully analyzed. In both, the same hypodiploid karyotype - which differed from the normal karyotype by a loss of 1 m chromosome - was observed constituting a stemline and a sideline, respectively. LEVAN (1961) reported a hypodiploid stemline with a loss of 2 m chromosomes in the haemorrhagic centre of a primary Rous rat sarcoma. The particular external or internal environment of tumours - e.g. focal tissue breakdown, infection, anoxia, nutritional inequalities - may favour the selection of specific karyotypic mutants. The findings may be incidental, but the loss of an m chromosome owing to the particular milieu in the interior of tumours may be comparable to the preferential loss of one st<sub>5</sub> chromosome in a transplanted Rous rat sarcoma after environmental changes (MITELMAN 1965).

#### Isozyme Patterns in Primary Rous Sarcomas (III)

Electrophoretic isozyme patterns of 9 enzyme systems were studied in normal tissues and in 11 primary Rous sarcoma virus-induced tumours of the W/Fu rat strain. This was undertaken to elucidate whether mutational activity produced by the oncogenic virus is reflected in the isozyme patterns of a number of well-known enzyme systems. Any differences in isozyme patterns among the tumours, and between the tumours and the normal tissue, would be attributable to the action of the virus. It was also of interest to elucidate whether differences in stemline karyotypes were correlated with differences in isozyme patterns.

The results indicated that all primary tumours showed largely similar deviations of the isozyme patterns when compared



with the tissue of origin (subcutaneous connective tissue). No obvious correlations were found between enzymatic properties and the chromosomal constitution; the karyotypic variation actually present among the material seems to have no significance for the variation of the isozyme patterns. This was also found in the experiments of HORI and SASAKI (1969) in which the G6PD patterns of 25 hepatomas induced by 3'-methyl-4-dimethylamino-azobenzene were largely similar, despite the fact that the tumours had different euploid and aneuploid stemlines.

#### Latent Period, Tumour Age and Chromosomes (II, V)

The 50 primary sarcomas appeared after a latent period ranging from 17 to 121 days. No correlation between the duration of the latent period and the chromosomal constitution of the tumours could be discerned: the mean latent period was roughly the same in the diploid sarcomas with and without sidelines and in the heteroploid sarcomas. The differences among the groups were not significant. The relationships short latent period and diploidy, and long latent period and polyploidy have been indicated by the results obtained from murine tumours induced by 20-methylcholanthrene (BIEDLER 1962; HANSTEEN and REFSUM 1969) and Freund's adjuvant (YOSIDA et al. 1968), in 3-methylcholanthrene-induced tumours in the golden hamster (NACHTIGAL et al. 1967), and in Rous virus-induced sarcomas in the Chinese hamster (KATO 1968). On the other hand, MARK (1967 b) found no difference in the latent period between normal diploid and tetraploid Rous tumours in the mouse, nor did the data of HELLSTRÖM et al. (1963) and JOHNSON et al. (1970) for polyoma virus-induced and plastic film-induced murine tumours, respectively, suggest any such relationship. Thus, no simple

correlation exists between the latent period and the chromosomal constitution of tumours. The differences actually found between different materials suggest that each carcinogenic agent affects each species in a particular way.

The 50 sarcomas were subjected to chromosomal analysis after a growth period of the tumours varying between 16 and 137 days. The mean tumour age of the diploid sarcomas lacking sidelines and those with sidelines was about the same (57 and 60 days, respectively), whereas the sarcomas with heteroploid stemlines were almost twice that age (103 days) at the time of fixation. By relating the tumour age to the chromosomal picture, it could be demonstrated that during the earliest stages the tumours not only had normal diploid stemlines but they consisted exclusively of normal diploid cells. As deviating cells started to appear, gradually forming sidelines, it follows that the proportion of normal cells decreases. An interesting observation was made by MARK (1969) when he was studying, in his material of Rous sarcomas in the mouse, the decrease with time in the number of diploid cells. He found that the correlation was non-linear, with a period of rapid loss of the diploid cells. The same phenomenon was apparent in the present material and can also be seen in the Chinese hamster material of KATO (1968, Fig. 15). Evidently, once the evolution has reached a critical point, the diploid cells are lost at an accelerated rate. This non-linear process was confirmed by the results of the transplantation studies (V) in which each individual tumour could be followed through different periods with samples taken on 8-16 occasions. It could be demonstrated further that the duration of the heteroploid population shift was determined mainly by the initiation period. An interesting parallel to the loss of normal diploid cells from the tumour cell population was demonstrated in human cervical carcinomas by GRANBERG (1971):

As long as the tumours remained in the pre-invasive stage, normal diploid cells predominated; but as soon as invasion started, normal diploid cells were no longer seen.

#### The Chromosomal Relationship Between Primary Sarcomas and Their Metastases (I, IV)

Comparative cytogenetic studies of primary tumours and their metastases are few and the results are contradictory. On the basis of microspectrophotometric measurements of the DNA content, RABOTTI (1959) first suggested a shift toward a higher level of ploidy in metastatic tumours. Using the same method, CHU and MALMGREN (1961) obtained similar results, whereas the results of MEEK (1961); STICH and STEELE (1962); INUI (1963); ATKIN et al. (1966); TAVARES (1968); ZANK and KRUG (1970) and ATKIN (1970, 1971) indicate that the level of ploidy of the primary neoplasm in general is repeated in the metastases. Changes in ploidy, when they occurred, were seen in both directions. In the majority of cases, chromosomal analyses have manifested identical or similar chromosome counts in both sites (GOODLIN 1962; MAKINO et al. 1964; MAEDA et al. 1965; INUI 1966; LUBS et al. 1966; ŠLOT 1967; WAKONIG-VAARTAJA and HUGHES 1967; NAPPI and CURCIO 1968; GRIPENBERG et al. 1969; MARK 1970; ATKIN 1971). Where differences were found between a primary tumour and its metastases, shifts were observed toward both lower (MAEDA et al. 1965; DURUMAN 1969; MARK 1970), and higher levels of ploidy (BAKER quoted in ATKIN 1970), as well as from a well-defined stemline to a wide distribution of chromosome counts without any clear mode (MAEDA et al. 1964). However, 4 of these 6 cases in which differences were observed between the two sites had received radiation or cytostatic therapy, the effects of which are impossible to predict. Detailed chromosome analyses of

single cases have further demonstrated that, irrespective of the chromosome numbers, the metastases have karyotypes similar to, or reflecting, those of the primary tumour (LUBS et al. 1966; ŠLOT 1967; NAPPI and CURCIO 1968; GRIPENBERG et al. 1969; PORTER et al. 1969; BAKER quoted in ATKIN 1970; MARK 1970; ATKIN 1971); but no systematic experimental study has been carried out relating the karyotypic status of primary tumours to their metastases.

In the present study, it was possible to analyze the chromosomes of 20 metastatic tumours together with the corresponding primary sarcomas. All metastatic tumours were either regional or para-aortic lymph node metastases. In 14 animals 1 metastasis was studied, and in 3 animals 2 different metastases of the same primary sarcoma could be analyzed. The results demonstrated a close karyotypic relationship on the one hand between the primary sarcomas and their metastases and on the other between different metastases of the same primary sarcoma. Heteroploid transformation of the primary sarcoma was not a prerequisite for the spreading process, and apparently normal diploid cells were capable of metastasizing. Not only stem- or sideline cells but also variant cells were potential metastatic stem cells. There was no selection of polyploid cells; whether or not diploid- near-diploid cells with any particular chromosome constitution were favoured could not be determined. However, the general pattern of the heteroploid evolution in the metastases was the same as in primary Rous rat sarcomas. Thus, hyperdiploidy was the most important heteroploid pathway, the second most important being pseudodiploidy, whereas hypodiploidy and polyploidy were of little or no importance.

Each primary sarcoma and its metastases were processed on the same occasion. Yet, 60 per cent of the metastases had he-



teroploid stemlines, as compared with only 18 per cent in the primary site. About 40 per cent of the primary sarcomas with a normal diploid stemline had sidelines, whereas sidelines were seen in 75 per cent of these secondary tumours. It thus appears that the chromosomal progression is accelerated in the lymph node metastases. Unfortunately, no haematogenic metastases could be studied. Hence, it cannot be stated whether this mechanism applies to other types of metastatic growth as well.

Karyologically, the findings in the metastases accorded with an acceleration of the non-random, stepwise changes observed in the primary sarcomas. As in that material, 1 extra  $t$  or  $st_3$  or both were observed in about 75 per cent of the deviating karyotypes. Their distribution, however, was different. The trisomic karyotypes with 1 extra  $t$ , which dominated the deviating stem- and sidelines and variant cells of the primary sarcomas, were only seen in about 10 per cent of the heteroploid metastatic stem- and sidelines, whereas the frequency of stem- and sidelines with 1 extra  $st_3$  had increased from 24 to 68 per cent. In addition to the extra  $t$  and  $st_3$ , a threefold increase of stem- and sidelines possessing 1 extra  $st_5$  chromosome was noticed in the metastatic tumours (see p. 24). The accelerated progression in the metastases is further stressed by the considerable increase of structural rearrangements: thus, the frequency of markers among the heteroploid stem- and sidelines was almost twice that in the primary sarcomas (45 and 24 per cent, respectively).

The chromosomal deviations in the 20 metastatic tumours were tested statistically in a similar way as the primary sarcomas. Significant, positive mean deviations were obtained for chromosome type  $st_3$  among stem- and sidelines with and without markers. There was a significant positive value for chromosome



type t among stem- and sidelines without markers and a corresponding high negative value for this chromosome type among stem- and sidelines with markers. Thus, similar results were obtained as in the primary sarcomas.

As for all virus-induced tumours, it might be argued that the metastases represent new inductions. Though this possibility cannot be wholly excluded, AHLSTRÖM and JONSSON (1962) found it unlikely for several reasons: 1) The rats which were killed early never exhibited tumour nodules in the lungs or lymph nodes, whereas growths in these organs were more or less the rule in animals allowed to live longer. 2) The nodules in lungs and lymph nodes were always much smaller than the tumours at the site of injection and showed the same location as those usually seen on dissemination of tumour cells. 3) Disseminated sarcoma cells were frequently observed in the lymphatics and small vessels. The generally close karyotypic relationship, and particularly the three cases with similar markers in both the primary and the secondary lesions, no doubt support the concept of metastasization.

#### The Chromosomal Progression During Early In Vivo Transplantation (V)

In order to test the hypothesis of sequential karyotypic changes, to elucidate whether additional steps could be discovered, and to find out to what extent the karyotypic changes in different series of passages of the same tumour were predetermined, the following experimental system was employed: Two primary sarcomas with a normal diploid stemline and lacking deviating cells were selected. From each tumour five and six parallel series of passages, respectively, were established. In each series the tumour was transplanted to app-

roximately 20 succeeding generations of rats. The chromosomes were studied intermittently in each series, usually every other passage. In all, 121 tumours were analyzed chromosomally.

During transplantation, all series of both tumours manifested a heteroploid transformation. The following steps could be demonstrated in this evolution:

1. the formation of single hyperdiploid variant cells;
2. the development of a hyperdiploid sideline from these cells;
3. replacement of the normal diploid stemline by the sideline, either directly or preceded by modifications of the sideline;
4. modifications of the heteroploid stemline, including shifts of the stemline category.

In both tumours the early progression displayed similar non-random and sequential karyotypic characteristics like those observed in the primary and the metastatic sarcomas: gains of chromosome types  $t$  and  $st_3$ . In addition, a third step - i.e. a gain of 1  $st_5$  chromosome - only discernible in the primary and metastatic tumours could be clearly demonstrated. From this point on, the karyotypic changes observed, numerical and structural, manifested no obvious pattern.

Among all series of passages derived from the same primary sarcoma, the earliest deviating cells had exactly the same karyotype. Two possible explanations can be offered:

1. The deviating cells originated de novo in the different series. The initial step in the heteroploid evolution of each tumour would then be predetermined with a specificity comprising not only the direction, i.e. hyperdiploidy, but also the karyotypic change.

2. The deviating cells originated in and were present already in the primary sarcoma, but in a proportion not detectable by counting 100 cells. By a selective advantage of this particular karyotype, these cells gradually increased in number and were observed as single deviating cells in passages 2-7 of the different series.

Neither of these two hypotheses can be proved at present. The likelihood, however, that the sequence of events that produced the conspicuous marker in all series of one of the sarcomas would occur repeatedly and independently in different cells must be considered as very small. Accordingly, an explanation in which such a mechanism is a prerequisite must be regarded as less probable. The question is of considerable theoretical importance and will be studied further by single-cell inoculations.

A similar comparative chromosomal investigation of the early in vivo progression in parallel series from the same primary tumour has not, to my knowledge, previously been published. Disregarding long-term transplanted experimental neoplasms which have undergone profound numerical and structural rearrangements (for review, see MAKINO 1957; LEVAN 1959; RUTISHAUSER 1963; JEAN and BOIS 1967), the information available on the early progressional changes in other types of rat tumours with identical aetiology is scarce. Information is, however, available about the early progression in 3 other in vivo transplanted Rous rat sarcomas (LEVAN 1961). The results of these original observations agree with the findings of the present study. Thus, all three early passages, 1, 1 and 5, respectively, exhibited an evolution towards stemlines with 43 chromosomes through a gain of 1 t chromosome. It is also interesting that another RSV-SR-induced rat sarcoma carried in serial pas-

sage since 1961, and also originally studied by LEVAN (1961), apart from numerous other rearrangements, still shows (MITELMAN, unpublished) two of the non-random changes, already observed in the primary tumour and in the intervening passages (KATO et al. 1964): one extra t and one extra st<sub>5</sub> chromosome.

In this context it should be mentioned that AL-SAAD I and BEIERWALTES (1967), AL-SAAD I (1968) and WU et al. (1971) reported a consistent chromosome change in transplanted thyroid rat tumours that had developed from goiters induced by iodine-deficiency. The progression to an anaplastic, autonomous tumour was correlated with a loss of one particular chromosome, identified by the authors as m<sub>2</sub> according to the nomenclature of the present work. This preferential loss was observed already in the evolution of the goiters of rats fed iodine deficient (AL-SAAD I and BEIERWALTES 1966).

#### Chromosomes and Histopathology (I, II, IV, V)

All primary, metastatic and transplanted tumours that were subjected to chromosome analysis were also examined histologically. All these tumours were identified as fibrosarcomas with varying degree of maturity. Three histologic classes could be distinguished: (1) highly differentiated fibrosarcomas, (2) spindle cell sarcomas, and (3) anaplastic round cell sarcomas with giant cells. The criteria predominantly applied in this classification were the degree of cellular differentiation and the degree of collagen production. Other histologic features proved less reliable because they lacked sufficient specificity; small necrotic and haemorrhagic areas were found in all types, and no sure difference was observed as regards the mitotic rate. When uncertainty of classification aro-

se, it always concerned neighbouring classes, and such tumours were assigned to the less differentiated class. The classification was performed without knowledge of the chromosomal data.

No relationship could be established between any particular chromosome number or karyotype and the histopathologic picture of the tumours, but the same trend was found in primary, metastatic and transplanted sarcomas: the tumours with a normal diploid stemline lacking sidelines were highly differentiated fibrosarcomas. With the appearance of heteroploid sidelines, the histologic picture changed to that of a spindle cell sarcoma, and the heteroploid transformation - irrespective of the stemline category - was associated with a change to an anaplastic sarcoma. It should be noted that the correlation was by no means an absolute one, and in many instances during in vivo transplantation the change in histologic maturity preceded or lagged behind the chromosomal changes by one or a few passages. The trend, however, was obvious: the chromosomal progression from normal diploidy to heteroploidy was associated with a histologic dedifferentiation. A similar relationship was found in RSV-induced sarcomas of the mouse (MARK 1968). The highly differentiated fibrosarcomas of the Chinese hamster, however, were correlated with one specific karyotype, viz. trisomy No. 6 (KATO 1968). The results of other attempts to correlate the chromosome picture with histology (BANERJEE and BATES 1966; HORI and SASAKI 1969; JOHNSON et al. 1970) agree insofar as highly anaplastic tumours tended to deviate in the tetraploid direction. It should be remembered, however, that two of these observations were made on murine tumours and that in the mouse tetraploidy is the most common progressional pathway. The association with anaplasia may accordingly have been coincidental.



## DISCUSSION AND CONCLUSIONS

In the table on the next page, pertinent data are listed on other primary experimental rat neoplasms studied chromosomally. As can be seen, no other virus-induced, solid rat tumour has been examined so far. Thirteen thymic leukaemias induced by the Gross virus (KIRSTEN and DOMINGUEZ 1966) had normal diploid stemlines and a hyperdiploid variation, but karyotypic details of these cells were not reported. Also one leukaemia induced by the Masurenko virus (FICHIDJIAN et al. 1964) had a normal diploid stemline, but with hypodiploid variation. Although karyotypic data in many cases are only fragmentary, the normal diploid karyotype also dominates in all other tumour types whether they are induced by chemical, hormonal or physical agents or are of spontaneous origin. In the total material, as in the present Rous material, hyperdiploidy is second in frequency, whereas hypodiploidy and polyploidy are of minor importance. It should be noted that within the hyperdiploid group, the trisomic number predominates. A review of the literature on primary experimental neoplasms (MITELMAN, unpublished) has further revealed that, irrespective of the species used or the inducing agent, the most common change from the normal is, in fact, a gain of just one chromosome. Also spontaneous neoplasms - both solid tumours and leukaemias - in different species, are dominated by the normal diploid number; the trisomic number being the dominating abnormality. The results of the present investigation and the abovementioned facts suggest that all neoplasms are initially comprised of normal diploid cells and that the deviation from the normal karyotype is initiated by the formation of a trisomic karyotype, whereas the further progression is determined by other factors, such as the inducing agent, the species used and environmental factors.

Percentage distributions of stemlines or modal numbers in experimental rat neoplasms studied in direct fixations or primary cultures

| Tumour type                       | Inducing agent                                   | Number of tumours | Stemline number of Hypo-diploid | Diploid | Hyper-diploid | ± 3x | ± 4x | ?  | Reference                      |
|-----------------------------------|--------------------------------------------------|-------------------|---------------------------------|---------|---------------|------|------|----|--------------------------------|
| Hepatoma                          | 3-methyl-4-dimethyl-aminobenzene                 | 19                | —                               | 68      | —             | 21   | 11   | —  | Hori and SASAKI 1969           |
| "                                 | 4-dimethylaminobenzene                           | 9                 | 44                              | 22      | 22            | —    | 11   | —  | Yosida and Ishihara 1956, 1957 |
| "                                 | "                                                | 6                 | —                               | 33      | —             | —    | —    | 67 | Bayreuther 1960                |
| "                                 | "                                                | 5                 | —                               | 20      | —             | 20   | 20   | 40 | Kinosita 1958                  |
| "                                 | "                                                | 3                 | —                               | —       | —             | 100  | —    | —  | Gläss 1960                     |
| "                                 | N-2-fluorenylacetanide                           | 8                 | —                               | 100     | —             | —    | —    | —  | Becker et al. 1971             |
| "                                 | Diethylnitrosamine                               | 1                 | —                               | 100     | —             | —    | —    | —  | Grover and Fischer 1971        |
| Mammary carcinoma                 | 3-methylcholanthrene                             | 10                | —                               | 100     | —             | —    | —    | —  | Majumdar and Rees 1970         |
| "                                 | 2-acetylaminofluorene                            | 6                 | —                               | 67      | —             | —    | —    | 33 | Bayreuther 1960                |
| "                                 | 3,4-benzpyrene                                   | 4                 | —                               | 50      | —             | —    | —    | 50 | Bayreuther 1960                |
| "                                 | 20-methylcholanthrene                            | 3                 | —                               | —       | 33            | 67   | —    | —  | Talukder and Sharma 1969       |
| "                                 | Iron dextran (Imferon®)                          | 2                 | —                               | —       | —             | 50   | 50   | —  | Davies (in Koller 1960)        |
| "                                 | β-naphthyl-di-(2-chloroethyl)-amine              | 1                 | —                               | —       | —             | 100  | —    | —  | Koller 1953                    |
| "                                 | Spontaneous                                      | 1                 | —                               | 100     | —             | —    | —    | —  | Wakong 1960                    |
| Rhabdomyosarcoma                  | Nickel sulfide                                   | 2                 | —                               | 100     | —             | 50   | —    | 50 | Basrur and Gilman 1963         |
| Thymic lymphosarcoma              | Mg-deficiency                                    | 5                 | —                               | 100     | —             | —    | —    | —  | Jean and Bois 1967             |
| Pituitary tumour                  | Oestrogen                                        | 14                | 14                              | 79      | 7             | —    | —    | —  | Waelbroeck-Van Gaver 1969      |
| Leukaemia                         | Gross virus                                      | 13                | —                               | 100     | —             | —    | —    | —  | Kirsten and Dominguez 1966     |
| "                                 | Masarengo virus                                  | 1                 | —                               | 100     | —             | —    | —    | —  | Fichidjian et al. 1964         |
| "                                 | 7,12-dimethylbenz (a) anthracene                 | 93                | 2                               | 47      | 48*           | 1    | 3    | —  | Kurita et al. 1968, 1969       |
| "                                 | "                                                | 8                 | —                               | 75      | 13            | —    | 13   | —  | Rees et al. 1970               |
| "                                 | 6,8,12- and 7,8,12-trimethylbenz (a) anthracene  | 64                | 2                               | 83      | 11            | 5    | —    | —  | Sugiyama and Brillantes 1970   |
| "                                 | 9,10-dimethyl-1,2-benzanthracene                 | 2                 | 50                              | —       | 50            | —    | —    | —  | Fichidjian et al. 1964         |
| "                                 | Methylcholanthrene                               | 12                | —                               | 100     | —             | —    | —    | —  | Nowell et al. 1963             |
| "                                 | 3-methylcholanthrene                             | 6                 | —                               | 100     | —             | —    | —    | —  | Moloney et al. 1965            |
| "                                 | X-ray                                            | 7                 | —                               | 100     | —             | —    | —    | —  | Dowd et al. 1964               |
| "                                 | Spontaneous or 3-methylcholanthrene and/or X-ray | 17                | —                               | 65      | —             | —    | —    | 35 | Moloney et al. 1969            |
| "                                 | Spontaneous                                      | 4                 | —                               | 50      | 50            | —    | —    | —  | Moloney et al. 1970            |
| Total and mean percentage values: |                                                  | 326               | 3                               | 65      | 19            | 5    | 3    | 5  |                                |

\* Including pseudodiploid S

It is of great interest that in the three primary neoplasms studied in species with a well-differentiated chromosome complement and also investigated in greatest detail in the literature, the deviating cells exhibit clear non-random patterns. As in the present material, these karyotypic changes are most pronounced among the trisomic cells. In addition to the pattern of the RSV-induced sarcomas of the Chinese hamster (KATO 1968) referred to earlier, SUGIYAMA et al. (1967) and KURITA et al. (1968, 1969) found a distinctive abnormality - trisomy of the longest t chromosome ( $t_1$ ) - in primary rat leukaemia induced by pulse doses of 7,12-dimethylbenz(a)anthracene (7,12-DMBA). About 30 per cent of the leukaemic rats had stemlines with this trisomy, which was also present in about 20 per cent of the non-modal cells. These findings were confirmed by REES et al. (1970), and further it was found that the leukaemia cells with the trisomy differed in several biologic and haematologic characters from the leukaemia cells with a normal karyotype (SUGIYAMA et al. 1969). The same abnormality was present also in rat leukaemias induced by the closely related hydrocarbon carcinogens 6,8,12- and 7,8,12-trimethylbenz(a)anthracene (SUGIYAMA and BRILLANTES 1970).

Chromosome analyses of primary 7,12-DMBA-induced rat sarcomas are in progress in our laboratory. So far, 12 sarcomas have been studied (MITELMAN and LEVAN 1972): 5 had normal diploid stemlines, 4 without sidelines; 5 were hyperdiploid; and 2 were tetraploid. It is of considerable interest that the chromosome variation in the development of the heteroploid stem- and sidelines exhibited almost identical features as those described in the 7,12-DMBA-induced leukaemias. Thus, all deviating stem- and sidelines in the diploid region showed a gain of the longest terminal chromosome ( $t_1$ ). This particular trisomy was also found in single variant cells of 3 of 4 sarcomas with a normal diploid stemline without sidelines, and in 1 of the 2 tetraploid sarco-

mas. Altogether, cells with the  $t_1$  trisomy were observed in 10 out of the 12 sarcomas. Furthermore, 1 extra m chromosome - the second common change in the 7,12-DMBA-induced leukaemias discussed above - was found in 7 sarcomas: as part of the stem- and sideline karyotypes in 3 and as variant cells in 4. Thus, irrespective of the nature of the transformed cell, blood-forming cell or fibroblast, 7,12-DMBA induces the same karyotypic changes, distinctively different from the changes induced by the Rous virus. If this mechanism is universal, it might explain the apparent lack of chromosomal patterns in many malignancies of the same histologic type, but presumably with different aetiology.

#### GENERAL SUMMARY

The present investigation of the chromosomes of Rous virus-induced sarcomas in the rat has given the following main results:

1. The chromosomal spectrum of the primary sarcomas was dominated by tumours with a normal diploid stemline. Hyperdiploidy was the most important evolutionary pathway, and within this group the trisomic number predominated. The second common progressional pathway was pseudodiploidy, whereas hypodiploidy and polyploidy were of minor importance.

2. There were indications that the primary sarcomas initially were comprised of exclusively normal diploid cells. With increasing tumour age, the normal diploid cells decreased rapidly by a non-linear process.

3. The karyotypic variation of the heteroploid evolution was non-random and sequential. There were indications that the initial change in each tumour was predetermined with a specificity not only for the general direction of the heteroploid pathway but also for the karyotype.

4. Chromosomal analyses of different regions of the same primary sarcoma were compatible with the concept that progression is monoclonal, i.e. originating from a single mutant cell.

5. The primary sarcomas manifested largely similar deviations of the isozyme patterns when compared with the tissue of origin.

6. A close karyotypic relationship was demonstrated between primary sarcomas and their metastases. The essential difference between them was an accelerated chromosomal progression in the secondary tumours.

7. The chromosomal progression in primary, metastatic and transplanted sarcomas was related to the histopathologic picture of the tumours: the progression from normal diploidy to heteroploidy was associated with a histologic dedifferentiation.



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